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Pezicula chiangraiensis sp. nov. from Thailand

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Abstract—A sexual morph of a new species, *Pezicula chiangraiensis*, was collected on bark of decaying wood in Chiang Rai Province, Northern Thailand. Morphologically it is closely related to *P. cinnamomea* but differs by its ascospores having a gelatinous sheath; in culture it produces a sporodochium-like asexual morph. Phylogenetic analysis of combined ITS, LSU, and RPB2 sequence data confirmed that *P. chiangraiensis* is distinct from other *Pezicula* spp. The new species is described, illustrated, and compared with similar taxa.

Key words—*Cryptosporiopsis*, *Dermateaceae*, discomycetes, phylogeny, taxonomy

Introduction

Pezicula Tul. & C. Tul. (*Leotiomycetes, Helotiales*, *Dermateaceae*), a widespread discomycete genus typified by *Peziza carpinea* Pers., was established by Tulasne & Tulasne (1865). Verkley (1999) accepted 26 species in *Pezicula*.

Subsequently, two new species and nine new combinations have been added to the genus (Johnston et al. 2014, Yuan & Verkley 2015, Zhong et al. 2001).

Pezicula species are characterized mainly by brightly coloured apothecia, which occur on the bark of woody plants (Ooki et al. 2003). The apothecia are frequently sessile, erumpent, and occasionally short-stalked with circular discs (Verkley 1999). The disc, initially concave and surrounded by slightly elevated margins, later becomes convex in most species (Verkley 1999). Most *Pezicula* species have 8-spored, clavate, or cylindric-clavate inoperculate asci. Ascospores vary in shape from broadly ellipsoid to elongately ellipsoid to allantoid or fusoid (Ooki et al. 2003, Verkley 1999).

Cryptosporiopsis has been reported as the asexual morph of *Pezicula* and 20 of the 26 *Pezicula* species accepted by Verkley (1999) are known to have *Cryptosporiopsis* asexual morphs. *Cryptosporiopsis* species produce sporodochial conidiomata, and most produce both macroconidia and microconidia that form on different conidiophores (Verkley 1999).

Pezicula and *Cryptosporiopsis* species are generally known as plant pathogens (Ooki et al. 2003). One *Cryptosporiopsis* species causes twig rot of cherry (Koiwa & Yamada 1996), and *C. corticola* (Edgerton) Nannf., the asexual morph of *Pezicula corticola* (C.A. Jørg.) Nannf., is reported to cause bull's-eye rot of Japanese pear. *Pezicula cinnamomea* (DC.) Sacc. causes stem canker of apple and pear (Nitta et al. 2002). *Pezicula* species are also endophytes (Kowalski & Kehr 1996). They play an important role in natural pruning that helps to produce a clean bole by decaying and shedding of dead branches (Kowalski & Kehr 1996). Species in this genus predominantly colonize woody twigs, although some occur on herbaceous plants (Gené et al. 1990, Kowalski & Bartnik 1995, Kowalski et al. 1998, Sankaran et al. 1995, Zhong et al. 2001). Most *Pezicula* species have been recorded from temperate and boreal forests of the northern hemisphere (Verkley 1999), with only a few species described from the southern hemisphere (Sankaran et al. 1995, Zhuang & Korf 1988).

Here we provide detailed morphological descriptions of sexual and asexual morphs of a new species, *Pezicula chiangraiensis*, and morphological comparisons with other closely related taxa. We also provide a multi-gene phylogenetic tree for *Pezicula* to infer the phylogenetic relationships of *P. chiangraiensis* with other *Pezicula* spp.

Materials & methods

A collection of *Pezicula* was made in Chiang Rai Province, Northern Thailand, in January 2015. Macroscopic and microscopic characters of the specimen were recorded. A Motic SMZ-168 stereomicroscope was used to observe the structure of the apothecia. Sections of apothecia were made by hand with a razor blade and mounted in water

and preserved in lacto-glycerol. A Nikon ECLIPSE 80i compound microscope was used to observe microscopic characters. Indian ink was added to detect the presence of mucilaginous sheaths. The amyloidity of asci was tested using Meltzer's reagent. Photomicrography was carried out with a Canon 450D digital camera fitted to the microscope. Measurements of apothecia, paraphyses, asci, and ascospores were made from material mounted in water. Measurements were made with the Taro soft (R) Image Frame Work v. 0.9.7 program and images used for figures were processed with Adobe Photoshop CS6 software (Adobe Systems).

Table 1 *Loramyces* and *Pezicula* species used in the phylogenetic analysis. New sequences are in bold.

Species	Culture no.	GenBank accession no.		
		ITS	LSU	RPB2
L. macrosporus	AFTOL-ID 913	JN033383	DQ470957	DQ470907
P. acericola	CBS 239.97	KF376154	KR858884	KF376214
	CBS 245.97	KF376153	KR858889	KF376213
P. aurantiaca	CBS 201.46	KF376150	KR858893	KF376210
P. californiae	CBS 124805	KR859104	KR858895	KR859332
	CBS 124819	GU973504	GU973597	–
P. carpinea	CBS 324.97	KF376156	KR858896	KF376160
	CBS 921.96	KF376155	KR858898	KF376159
P. chiangraiensis	**MFLUCC 15-0170**	**KU310621**	**KU310622**	**KU310623**
P. cinnamomea	CBS 239.96	KF376102	KR858915	KF376165
	CBS 625.96	KF376104	KR858943	KF376164
	CBS 240.96	KF376105	KR858916	KF376163
	CBS 626.96	KF376103	KR858944	KF376162
P. corni	CBS 285.39	AF141182	–	–
P. corticola	CBS 259.31	AF141179	KR858956	–
	CBS 260.31	KR859165	KR858957	–
P. corylina	CBS 249.97	KF376106	KR858960	KF376161
P. diversispora	CBS 185.50	KR859170	KR858962	–
	CBS 282.47	KR859171	KR858963	–
P. eucrita	CBS 656.96	KF376144	KR858977	KF376208
	CBS 257.97	KR859177	KR858969	–
P. frangulae	CBS 778.96	KF376151	KR859001	KF376212
	CBS 100244	KF376152	KR858996	KF376211
P. livida	CBS 262.31	AF141180	–	–
P. melanigena	CBS 898.97	KR859211	KR859003	–
P. neosporulosa	CBS 724.95	KR859231	KR859023	–
	CBS 660.95	KR859228	KR859020	–
	CBS 101.96	KR859223	KR859015	–
P. ocellata	CBS 949.97	KF376149	KR859025	KF376215
P. pruinosa	CBS 292.39	AF141188	KR859026	–
P. rubi	CBS 253.97	KF376100	KR859042	KF376204
	CBS 593.96	KF376101	KR859045	KF376203
P. sporulosa	CBS 224.96	AF141172	KR859053	KF376201
	CBS 225.96	KF376107	KR859054	KF376202
P. subcarnea	CBS 203.46	AF141171	KR859059	–

Observation of cultures and asexual morph

A pure culture was obtained from single spore isolation as described by Chomnunti et al. (2014). Germinating spores were transferred to malt extract agar (MEA) and incubated at 25 °C for one week. Cultural characteristics, such as mycelium colour, shape and texture, were determined. After one week, hyphal tips were cut and transferred to new media. The cultures were incubated at 25 °C under light for one week and growth rate was measured. After one week, cultures on MEA were checked for asexual structures. Conidiophores, conidiogenous cells and conidia were observed and measured by phase contrast microscopy under 400× and 1000× optical magnifications. The type specimen was deposited in the Mae Fah Luang University Herbarium, Chiang Rai, Thailand (MFLU) and an ex-type culture in Mae Fah Luang University Culture Collection (MFLUCC). A Faces of Fungi number was obtained as described in Jayasiri et al. (2015).

DNA isolation, PCR, and sequencing

The fungal isolate was grown on potato dextrose agar (PDA) for 7–21 days at 25 °C. Total genomic DNA was extracted as described by Thambugala et al. (2015). Polymerase chain reactions (PCR) were carried out using three partial gene regions: ITS4 and ITS5 (White et al. 1990) for the internal transcribed spacer (ITS); LROR and LR5 (Vilgalys & Hester 1990) for the nuclear ribosomal large subunit (LSU); and fRPB2-5F and fRPB2-7cR (Liu et al. 1999) for RNA polymerase II (RPB2). The PCR mixtures (25 µL) contained ddH_2O (9.5 µL), PCR Master Mix (TIANGEN Co., China) (12.5 µL; 2×), DNA template (1 µL), each primer (1 µL; 10 µM). Amplification conditions for all regions comprised an initial denaturation step at 94 °C for 5 min and final extension step at 72 °C for 10 minutes. ITS amplification comprised 34 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and elongation at 72 °C for 1 minute. LSU and RPB2 amplification comprised 37 cycles of denaturation at 94 °C for 1 minute, annealing at 54 °C for 50 seconds, and elongation at 72 °C for 1 minute. The PCR products were viewed on 1% agarose electrophoresis gels stained with ethidium bromide. The PCR products were purified and sequenced at Invitrogen Biotechnology Co., Shanghai, China.

Sequence alignment and phylogenetic analysis

Newly generated sequences were subjected to a standard BLAST search of GenBank for rough identification. Eighty-nine sequences belonging to three gene regions (ITS, LSU, RPB2) from representative *Pezicula* species and the outgroup taxon *Loramyces macrosporus* Ingold & B. Chapm. were downloaded from GenBank (Table 1); these sequences were published by Abeln et al. (2000), Chen et al. (2016), Spatafora et al. (2006), and Verkley (1999). The newly generated sequences were deposited in GenBank (Table 1). The consensus sequences for each gene were aligned using MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html). The single alignment was improved manually where necessary using BioEdit (Hall 2004). The single gene alignments were concatenated into a combined dataset. Maximum likelihood phylogenetic analyses were performed in CIPRES web portal (Miller et al. 2009) using RAxML-HPC Black Box (8.2.4) tool (Stamatakis 2006). The combined gene analysis was carried out using default conditions. The best scoring tree was selected with a final likelihood value of −7794.684804. The resultant tree was viewed with FigTree v.1.4.0

(http://tree.bio.ed.ac.uk/software/figtree/). Maximum Likelihood bootstrap values ≥60% are given near the nodes (Fig. 1).

Molecular phylogeny

A backbone tree for the genera *Pezicula*, *Cryptosporiopsis*, and *Neofabraea* was generated using ITS sequence data (data not shown). Two basic clades, the *Pezicula–Cryptosporiopsis* clade and the *Neofabraea* clade, were identified in the backbone tree, and *Pezicula chiangraiensis* grouped with other *Pezicula* species within the *Pezicula–Cryptosporiopsis* clade. A single gene tree for the genus *Pezicula* was generated from ITS sequence data (data not shown). As the single gene tree shows several taxonomic disagreements, a combined ITS, LSU and RPB2 dataset comprising 34 strains of *Pezicula* species with *Loramyces macrosporus* (CBS 235.53) as outgroup was generated to determine the exact species placement of our strain (Fig. 1). The resulting tree supports our new isolate in a distinct sister clade to *Pezicula cinnamomea* with strong bootstrap support (93%).

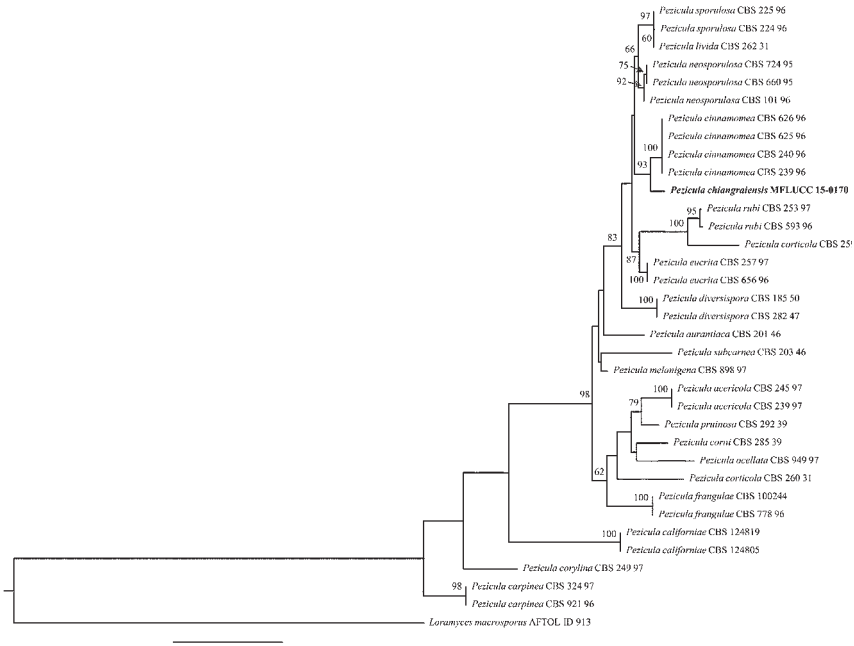
Pezicula sporulosa CBS 225 96
Pezicula sporulosa CBS 224 96
Pezicula livida CBS 262 31
Pezicula neosporulosa CBS 724 95
Pezicula neosporulosa CBS 660 95
Pezicula neosporulosa CBS 101 96
Pezicula cinnamomea CBS 626 96
Pezicula cinnamomea CBS 625 96
Pezicula cinnamomea CBS 240 96
Pezicula cinnamomea CBS 239 96
Pezicula chiangraiensis MFLUCC 15-0170
Pezicula rubi CBS 253 97
Pezicula rubi CBS 593 96
Pezicula corticola CBS 25
Pezicula eucrita CBS 257 97
Pezicula eucrita CBS 656 96
Pezicula diversispora CBS 185 50
Pezicula diversispora CBS 282 47
Pezicula aurantiaca CBS 201 46
Pezicula subcarnea CBS 203 46
Pezicula melanigena CBS 898 97
Pezicula acericola CBS 245 97
Pezicula acericola CBS 239 97
Pezicula pruinosa CBS 292 39
Pezicula corni CBS 285 39
Pezicula ocellata CBS 949 97
Pezicula corticola CBS 260 31
Pezicula frangulae CBS 100244
Pezicula frangulae CBS 778 96
Pezicula californiae CBS 124819
Pezicula californiae CBS 124805
Pezicula corylina CBS 249 97
Pezicula carpinea CBS 324 97
Pezicula carpinea CBS 921 96
Loramyces macrosporus AFTOL ID 913

Fig. 1. The best scoring RAxML maximum likelihood phylogenetic tree based on a combined ITS, LSU, and RPB2 sequence dataset. Strain/culture numbers are given following the taxon names. The newly generated sequences are in **bold**. Bootstrap support values for maximum likelihood >60% are given near the nodes. The tree is rooted with *Loramyces macrosporus* (CBS 235.53).

Taxonomy

Pezicula chiangraiensis Ekanayaka & K.D. Hyde, **sp. nov.** Plates 1, 2

Index Fungorum IF551789

Differs from other *Pezicula* species by possessing ascospores with a gelatinous sheath.

Type: Thailand, Chiang Rai Province, Kun Korn waterfall, 21 January 2015, A.H. Ekanayaka (**Holotype,** MFLU 15-3566; ex-type culture, MFLUCC 15-0170; GenBank KU310621, KU310622, KU310623).

Etymology: With reference to the province where the holotype was collected.

FacesofFungi: FoF 01667

Sexual morph: Saprobic on dead stems. Apothecia 800–870 × 230–280 µm (x = 826 × 253 µm, n = 10), arising singly or in small groups, sessile, slightly erumpent from the substrate, subsphaerical, urceolate, bright yellow when fresh, brownish yellow when dried. Disc convex. Receptacle yellow to light brown when fresh. Ectal excipulum variably thick, composed of large thin-walled yellow cells in a textura angularis. Medullary excipulum thin, not clearly distinguishable, composed of vertically arranged rows of yellow cells in a textura prismatica. Hymenium hyaline. Paraphyses 1.8–2.1 µm diam. (x = 2.0 µm, n = 20), numerous, filiform, obtuse, septate, branched, slightly enlarged at the apex. Asci 90–120 × 15–25 µm (x = 108 × 19 µm, n = 30), 8-spored, unitunicate, cylindric-clavate, short pedicellate, arising from croziers with conical and non-amyloid apices. Ascospores 18–22 × 8–10 µm (x = 20 × 9 µm, n = 40), partially biseriate, lower spores uniseriate, hyaline to grey, fusoid, gelatinous sheath present; immature spores non-septate and filled with guttules; mature spores 3-septate, aguttulate, and smooth-walled.

Asexual morph: Conidiomata sporodochium-like, produced in 7-day-old cultures, gregarious, dark brown. Conidiophores 11–12 × 5–6 (x = 11.5 × 5.8 µm, n = 20), branched near the base, septate, smooth-walled, composed various size cells. Conidiogenous cells holoblastic, cylindrical, smooth, hyaline. Conidia 23–29 × 11–12 µm (x = 25.2 × 11.2 µm, n = 20), cylindrical, straight, smooth with rounded apex and slightly truncate base, multi-guttulate or filled with granular cytoplasm, hyaline and aseptate when immature, pale brown and 1–3-transversely septate at maturity.

Culture characteristics: Ascospores germinating on MEA within 24 h. Colonies on MEA plates at 25–27 °C reaching 2 cm diameter within 1 week, circular, flat or effuse, with diffuse edge, medium honey-brown, grey aerial mycelium surrounded by pink mycelia, mycelium composed of 2–3 µm wide, septate, branched, smooth, hyaline hyphae, reverse dark brown to black.

Notes—*Pezicula cinnamomea* is morphologically similar to *P. chiangraiensis* in having apothecia, paraphyses, asci, and ascospores of a similar size and

Plate 1. *Pezicula chiangraiensis* (holotype, MFLU 15-3566), sexual morph: a. Substrate; b. Ascomata on wood; c. Ascoma; d. Cross section of ascoma; e. Vertical section of ascomal margin; f. Septate paraphyses; g–j. Short pedicellate asci; k. Germinated ascospore; l. Apex of mature ascus; m. Ascospore with gelatinous sheath; n–q. Fusoid ascospores. Scale bars: b = 500 µm; c, e = 100 µm; d = 300 µm; f, k = 50 µm; g–j = 40 µm; l, m = 20 µm; n–q = 10 µm.

shape, but differs by its pale luteous to cinnamon or red-brown apothecia, its transversely and longitudinally septate spores without gelatinous sheaths, its faster growth rate, and absence of diffusing pigment in culture (Ooki et al. 2003,

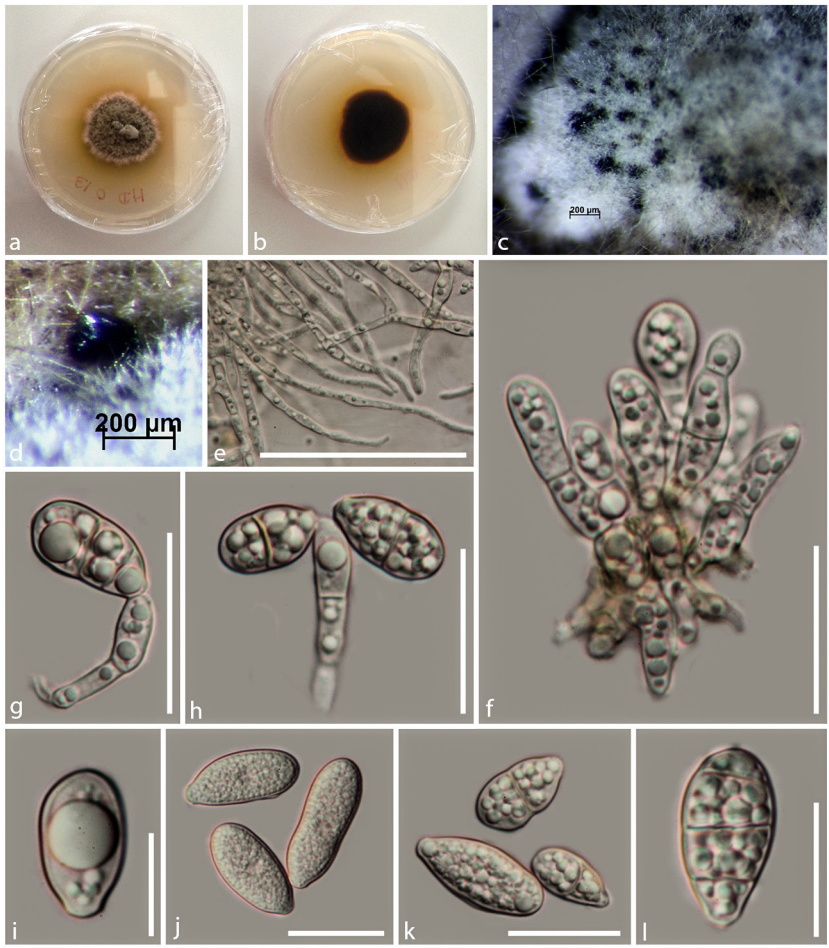

Plate 2. *Pezicula chiangraiensis* (MFLUCC 15-0170), asexual morph in culture: a. 7-day-old MEA colony from above; b. 7-day-old MEA colony from below; c. Conidiomata; d. Conidioma; e. Septate and branched hyphae; f. Fragment of small sporodochium-like conidioma; g, h. Different stages of conidiogenesis; i–l. Conidia at different stages. Scale bars: e = 50 µm; f, j–l = 20 µm; g, h = 30 µm; i = 10 µm.

Verkley 1999). *Pezicula aesculea* Kirschst. differs from *P. chiangraiensis* by its larger apothecia, asci, and ascospores; *P. amoena* Tul. & C. Tul. by its dark brown, thick-walled ascospores; *P. heterochroma* Verkley, *P. frangulae* (Pers.) Fuckel, *P. rubi* (Lib.) Niessl, *P. puberula* (E.J. Durand) Verkley, *P. crataegicola* (E.J. Durand) J.W. Groves, *P. myrtillina* (P. Karst.) P. Karst., and *P. grovesii* Wehm. by their short, stout stalked apothecia; *P. linda* Korf and

P. sepium (Desm.) Dennis by their asci with an amyloid ring; *P. tasmanica* W.Y. Zhuang & Korf by its positive reaction with Meltzer reagent after pretreating with KOH; *P. hamamelidis* J.W. Groves & Seaver by its broader apothecial base that is entirely hidden under the bark of its host substrate; and *P. melastomatis* Rehm by its smaller asci and ascospores (Verkley 1999). The asexual morphs of most *Pezicula* species produce both macro and microconidia (Verkley 1999), but microconidia were not observed in *P. chiangraiensis*.

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